Cell-cell interactions during osteoclastogenesis

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CHAPTER 4

IL-1β favors osteoclastogenesis via the supporting fibroblasts

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Abstract

Periodontitis leads to unwanted bone loss as a response to inflammatory compounds such as interleukin-1β (IL-1β). This excessive bone loss reflects both an increased osteoclast activity and formation of these cells. In this study we investigated how a short pre-incubation of periodontal ligament fibroblasts with IL-1β affected osteoclastogenesis. Fibroblasts were pre-incubated with IL-1β and/or dexamethasone (a commonly used anti-inflammatory compound) before being co-cultured with peripheral blood mononuclear cells (PBMCs). Pre-incubation with IL-1β (1-100 ng/ml) resulted in an increased number of adhered PBMCs. mRNA expression of intercellular adhesion molecule-1 (ICAM-1), macrophage colony stimulating factor (M-CSF) and IL-1β was highly increased. Pre-incubation with IL-1β also caused retraction of fibroblasts and increased the formation of $TRACP^+$ multinucleated cells.

Our data suggest that a short stimulation of the fibroblasts with IL-1β has a long lasting effect, leading to a significantly increased osteoclastogenesis. These results provide new insights for controlling excessive bone loss in periodontitis.

Introduction

Inflammation is often accompanied by an increased osteoclast-mediated bone resorption and a favored osteoclast formation. Multinucleated osteoclasts are formed through differentiation and fusion of hematopoietic mononuclear cells. These osteoclast precursors are recruited from the monocyte population and interact with supporting osteoblast-like cells such as periodontal ligament fibroblasts to receive the proper signals to further differentiate towards an osteoclast [1-3]. It has been shown that osteoblastic intercellular adhesion molecule-1 (ICAM-1) is involved in the adhesion between those two cell types [4] in such a way that ICAM-1 lacking osteoblast-like cells were not able to support osteoclastogenesis [5]. ICAM-1 on the osteoblast-like cell binds to its ligand leukocyte function associated antigen-1 (LFA-1) on the osteoclast precursor and facilitates the interaction of receptor activator of nuclear factor-κB (RANKL) with its receptor RANK on the osteoclast precursor. In conjunction with RANKL, macrophage colony-stimulating factor (M-CSF) is also an important cytokine that, through binding

to its receptor, c-fms, drives the osteoclast precursor towards the osteoclast lineage [6-8].

After adhesion, the osteoblast-like cells retract to create a space for the triggered osteoclast precursor which then migrates to the bone surface and fuses with other osteoclast precursors to form a multinucleated osteoclast. Direct cell-cell interaction between osteoclast precursors and osteoblast-like cells plays herein an important role [4,9].

It has been demonstrated that pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis-factor alpha (TNF-α) can modulate the expression of ICAM-1 in several cell types [10-13], including osteoblast-like cells [5], but whether this results in an increased adhesion of osteoclast precursors remains to be elucidated. The effects on mRNA expression are already visible within a short period of time (hours) after administration [14]. An increase in endothelial ICAM-1 leads to activation of these cells resulting in adhesion and transendothelial migration of circulating monocytes [15]. IL-1β can induce the expression of ICAM-1 and RANKL not only by endothelial cells but also by periodontal ligament fibroblasts [16,17]. These fibroblasts are known to support osteoclastogenesis [16,18]. Whether a short stimulation with IL-1β can also affect the later stages of osteoclast formation is not known.

To suppress inflammation, anti-inflammatory agents such as dexamethasone are often used. There is ample evidence that dexamethasone can inhibit IL-1β-induced expression of adhesion molecules and and directly temper the expression of IL-1β itself [12,19-21].

In this study we investigated whether a short stimulation of periodontal ligament fibroblasts with IL-1β prior to the addition of osteoclast precursors has an effect on the adhesion of the latter cells and their subsequent differentiation into TRACP+, multinucleated osteoclast-like cells. We further analyzed the effect of IL-1β on the mRNA expression of osteoclastogenesis-related genes at different time points. In addition, we examined whether the effects of IL-1β were influenced by the presence of dexamethasone.

Materials and methods

Cell cultures

Human periodontal ligament fibroblasts were retrieved from patients undergoing third molar extractions at the Department of Maxillofacial Surgery

(Academic Centre for Dentistry (ACTA)/VU University Medical Center (VUMC). The molars were obtained with informed consent and the use of periodontal ligament fibroblasts for this study was approved by the Medical Ethical Committee of the VU University. The fibroblasts were isolated according to previously described protocols [22].

Peripheral blood mononuclear cells (PBMCs) were isolated from a buffy coat (Sanquin, Amsterdam, The Netherlands) according to previously described protocols [4].

Periodontal ligament fibroblasts ($1.5x10^4$ cells per well) were seeded in a 48-wells plate (Costar, Cambridge, MA) for 2 days. Thereafter, the cells were stimulated for 6 hours with IL-1β (1, 10 and 100 ng/ml) in the absence or presence of dexamethasone (10^{-8}M) prior to co-culture with PBMCs. After stimulation, the different media were removed and all cell cultures were washed extensively with PBS. $5x10^5$ PBMCs per well were seeded on top of the fibroblasts. The co-cultures were further cultured for an additional 3 days (RNA analysis and adhesion analysis) or 21 days (osteoclast formation) in DMEM containing 10% FCS and 1% PSF.

Cultures used for RNA analysis were washed twice with PBS at the end of the culture period and stored in lysis buffer (Qiagen, Hilden, Germany) containing 1% β-mercaptoethanol at -80℃ until RNA extraction.

Cultures used for the quantification of adhesion were washed three times with PBS and cells were fixed in 4% formaldehyde in PBS for 10 min. Images were taken with a Leica phase contrast microscope (Leica, Wetzlar, Germany) and the PBMCs adhered to fibroblasts were counted.

RNA analysis and real-time quantitative PCR

RNA from cultured cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 100 ng RNA was used in the reverse transcriptase reaction which was performed according to the MBI Fermentas cDNA synthesis kit (Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers. A previously described protocol was used [4].

Real-time PCR was performed on an ABI PRISM 7000 (Applied Biosystems Foster City, CA). Primer sequences are listed in Table 1, all PCR efficiencies were comparable.

Table 1. Primers used for quantitative RT-PCR

Primer	Sequence 5' -> 3'	Amplicon length (bp)
ICAM-1:		
Forward	TgAgCAATgTgCAAgAAgATAgC	104
Reverse	CCCgTTCTggAgTCCAgTACA	
LFA-1:		
Forward	gAgCTggTgggAgAgATCgA	106
Reverse	gAggCgTTgCTgCCATAgA	
M-CSF:		
Forward	CCgAggAggTgTCggAgTAC	100
Reverse	AATTTggCACgAggTCTCCAT	
RANKL:		
Forward	CATCCCATCTggTTCCCATAA	60
Reverse	gCCCAACCCCgATCATg	
OPG:		
Forward	CTgCgCgCTCgTgTTTC	100
Reverse	ACAgCTgATgAgAggTTTCTTCgT	
IL-1β:		
Forward	CTTTgAAgCTgATggCCCTAAA	100
Reverse	AgTggTggTCggAgATTCgT	
PBGD:		
Forward	TgCAgTTTgAAATCATTgCTATgTC	84
Reverse	AACAggCTTTTCTCTCCAATCTTAgA	

ELISA assay

Measurements of M-CSF and IL-1β protein levels were performed using highly sensitive enzyme-linked immunoassay from R&D systems (Abingdon, United Kingdom) and Sanquin (Amsterdam, the Netherlands) respectively and according to the manufacturer's instruction. All ELISA measurements were performed on conditioned medium samples.

TRACP staining

After 3 weeks of culture, cells were fixed in PBS buffered 4% formaldehyde and stained for TRACP activity using the leukocyte acid phosphatase kit (Sigma). Nuclei were stained with diamidino-2-phenylindole dihydrochloride (DAPI).

Osteoclast quantification and fibroblast-free area assessment

Micrographs were taken from five fixed positions per well. For each micrograph, (i) the number of TRACP-positive multinucleated cells (cells with 3 or more nuclei) was counted and (ii) the surface area of the well devoid of fibroblasts was assessed.

Statistical analysis

One way-ANOVA of repeated measures was performed to assess whether there was a significant difference in mRNA expression, adhesion and osteoclast formation between groups. As a post-hoc test, a Bonferroni comparison between pairs of groups was used. A Kruskal-Wallis test followed by a Dunn's multiple comparison test was used to test significance in the protein gene expression data. In figures 1, 2, 3 and 4 results are expressed as means ± SEM. Differences were regarded significant when $p < 0.05$.

Results

IL-1β stimulates the number of adhered PBMCs to periodontal ligament fibroblasts

Stimulation of periodontal ligament fibroblasts with IL-1β prior to the addition of PBMCs led to an increase in the number of adhered PBMCs (Fig. 1A,B). A dose-dependency study revealed that all concentrations of IL-1β used (1, 10 and 100 ng/ml) significantly and dose-dependently augmented this number (Fig. 1B) (correlation analysis: $r=0.46$, $p= 0.03$). Comparing the number of adhered PBMCs in cultures with dexamethasone and with and without IL-1β showed that there was no significant effect of dexamethasone (Fig. 1C). Since a maximal response was found with 10 ng/ml IL-1β, all following experiments were performed using this concentration.

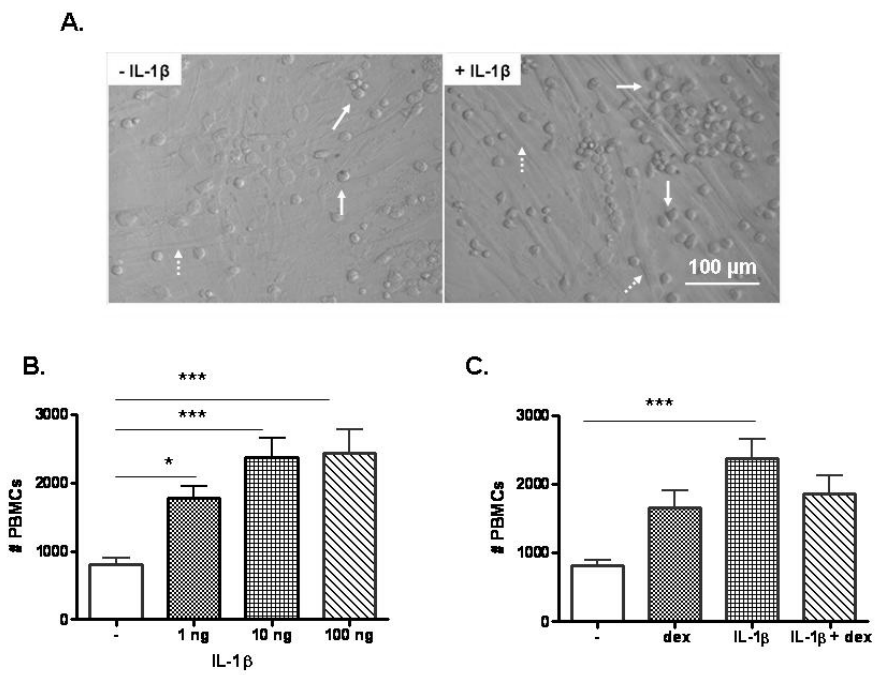

Figure 1. IL-1β stimulates adhesion of PBMCs to periodontal ligament fibroblasts.
A. IL-1β stimulated human periodontal ligament fibroblasts (dashed arrows) were, after six hours incubation with the pro-inflammatory cytokine, co-cultured with peripheral blood mononuclear cells (full arrows) for 3 days. Examples of PBMCs adhered to a confluent layer of control and IL-1β primed fibroblasts are shown. More adhered PBMCs were found in the IL-1β stimulated culture compared to the control. B. Adhesion of PBMCs to periodontal ligament fibroblasts was analyzed in the presence of various IL-1β concentrations. The total number of adhered PBMCs/well is depicted. C. The effect of dexamethasone (10^{-8}M; dex), with or without IL-1β (10 ng/ml), on the number of adhered PBMCs. The total number of adhered PBMCs/well is depicted for each culture condition. Data are expressed as mean number ± SEM in co-cultures of cells from five different donors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. For color figure see p. 159.

Expression of osteoclastogenesis-related genes by periodontal ligament fibroblasts increases after IL-1β stimulation

Effect after 6 hours

To investigate whether IL-1β alters gene expression by periodontal ligament fibroblasts, we analyzed the mRNA expression of several genes related to osteoclastogenesis in a mono-culture of the fibroblasts after incubation for

six hours with the pro-inflammatory cytokine and in the absence or presence of dexamethasone.

The expression of both ICAM-1 and M-CSF was augmented up to 30-fold after IL-1β stimulation (Fig. 2A). The addition of dexamethasone had no significant effect on the expression of these genes and also no effect was found when dexamethasone was added alone. The expression of IL-1β (Fig. 2A) or RANKL (Fig. 2B) was not significantly different in the presence of IL-1β, with or without dexamethasone. OPG mRNA expression was induced by IL-1β and this induction was inhibited by dexamethasone (Fig. 2B).

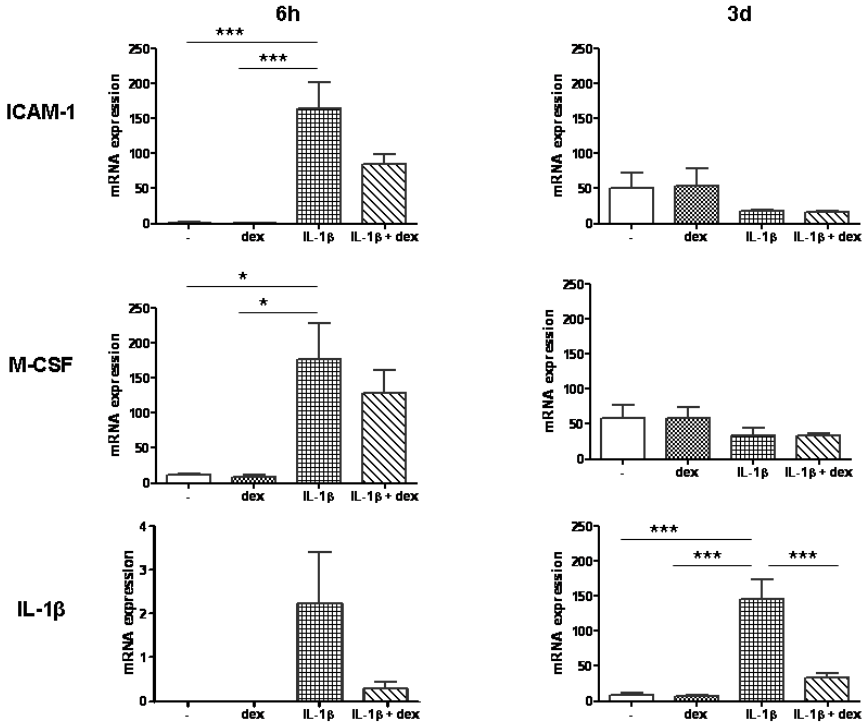

Figure 2A. mRNA expression of osteoclastogenesis related genes by periodontal ligament fibroblasts increases after IL-1β stimulation

Human periodontal ligament fibroblasts were incubated for six hours (left column) with IL-1β (10 ng/ml) and/or dexamethasone (10^{-8}M; dex) and gene expression of osteoclastogenesis related genes ICAM-1, M-CSF and IL-1β was analyzed and compared to the gene expression in their subsequent co-cultures with peripheral blood mononuclear cells (PBMCs) after 3 days (right column) of culture. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are expressed as mean expression ± SEM in mono-cultures and co-cultures of cells from five different donors

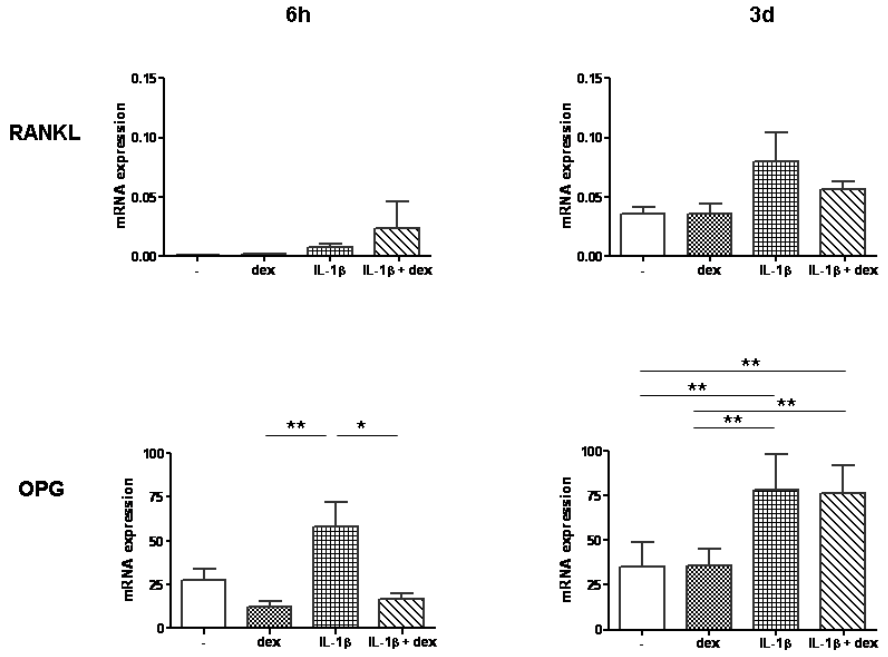

Figure 2B. mRNA expression of osteoclastogenesis related genes by periodontal ligament fibroblasts increases after IL-1β stimulation
Human periodontal ligament fibroblasts were incubated for six hours (left column) with IL-1β (10 ng/ml) and/or dexamethasone (10^{-8}M; dex) and gene expression of osteoclastogenesis related genes RANKL and OPG was analyzed and compared to the gene expression in their subsequent co-cultures with peripheral blood mononuclear cells (PBMCs) after 3 days (right column) of culture. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are expressed as mean expression ± SEM in mono-cultures and co-cultures of cells from five different donors.

Effect after 3 days

In order to obtain insight into the priming effects of IL-1β, we also analyzed the mRNA expression of the genes described above in the co-culture after three days. This revealed that the IL-1β-induced increased expression of ICAM-1 at the six hour time point was no longer present at the 3 day time point (Fig. 2A). A similar pattern was found for the mRNA expression of M-CSF. In contrast, at the 3 day time point IL-1β was highly increased in the co-culture with IL-1β stimulated fibroblasts; the level being approximately 150-fold higher compared to the controls (Fig. 2A). Interestingly, this increased expression was completely inhibited by dexamethasone (Fig. 2A;

note the different scale of the Y-axis of the two IL-1β graphs). mRNA expression of RANKL was increased in the co-culture, but no additional effect of IL-1β and/or dexamethasone was found (Fig. 2B). OPG mRNA expression was strongly up-regulated In the presence of IL-1β and this could not be reversed by the addition of dexamethasone (Fig. 2B). Calculating the RANKL/OPG ratios for the different culture conditions revealed that this ratio was not affected by IL-1β after 6h (-:0.013; dex: 0.126, IL-1β: 0.132, IL-1β + dex: 1.392) nor after 3 days (-: 1.0; dex: 0.996, IL-1β: 1.017, IL-1β + dex: 0.729). Though, this ratio strongly increased after 3 days compared to 6h in all the cultures except the one stimulated with IL-1β in combination with dexamethasone.

In addition, we investigated the effects of IL-1β and dexamethasone on the expression of osteoclastogenesis-related proteins after three days of co-culture (Fig. 3). Dexamethasone increased M-CSF protein expression whereas no significant effect of IL-1β was found. The concentration of IL-1β in conditioned medium was highly up-regulated when the periodontal ligament fibroblasts were pre-incubated with IL-1β (Fig. 3). No significant effect of dexamethasone was found on the IL-1β-induced IL-1β protein expression (Fig. 3).

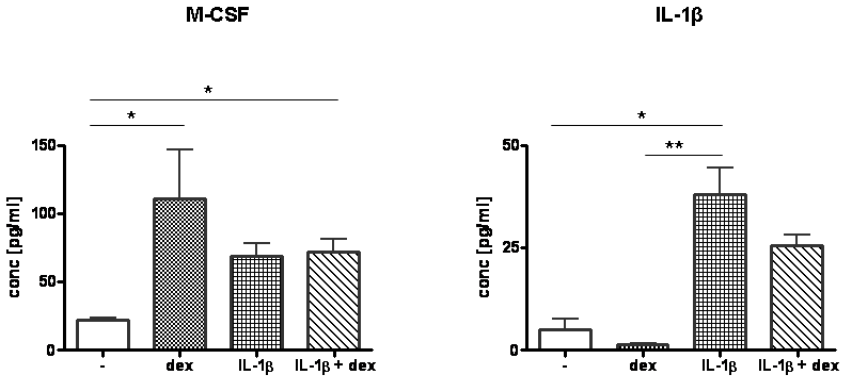

Figure 3. Protein expression of M-CSF and IL-1β after 3 days in a co-culture of periodontal ligament fibroblasts and PBMCs
Human periodontal ligament fibroblasts were pre-incubated for six hours with IL-1β (10ng/ml) and/or dexamethasone (10^{-8}M; dex) before co-cultured with peripheral blood mononuclear cells (PBMCs). Protein expression of M-CSF and IL-1β was analyzed after 3 days of co-culture by ELISA and data are expressed as mean expression ± SEM in medium of cultures of cells from five different donors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

A short stimulus of IL-1β causes an increased retraction of periodontal ligament fibroblasts and subsequent formation of osteoclast-like cells

Based on the significant changes in mRNA expression after a relatively short (6h) incubation with IL-1β, we wondered whether this could also affect the fibroblasts at a later stage during osteoclast formation. Since it has been shown that the retraction of osteoblast-like cells is essential for the migration of osteoclast precursors to the bone surface [18], we analyzed the surface area where the fibroblasts had retracted (fibroblast-free area) in conjunction with the number of osteoclasts formed under the different culture conditions (Fig. 4). Examples of the different cultures are shown in Fig. 4A.

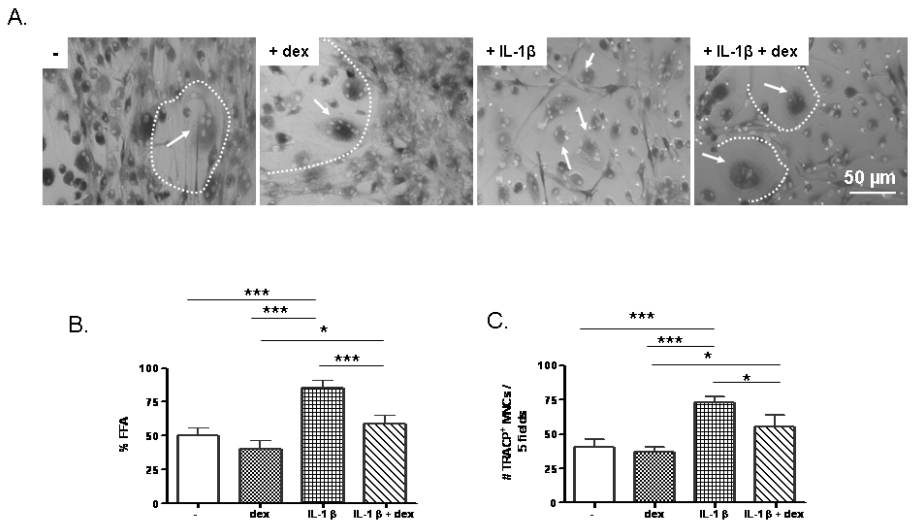

Figure 4. IL-1β stimulated fibroblasts favors retraction and the subsequent formation of osteoclasts

A. The retraction of fibroblasts (fibroblast free areas are enclosed with a dashed line) and the subsequent formation of TRACP-positive, multinucleated cells (full arrows) was analyzed in co-cultures in the presence or absence of IL-1β (10ng/ml) and dexamethasone (10^{-8}M; dex). B. The surface area devoid of fibroblasts (fibroblast free areas, FFA) was analyzed under the different culture conditions (IL-1β, 10ng/ml; dex 10^{-8}M). The surface area without fibroblasts (% FFA) was increased in the presence of IL-1β compared to controls C. The number of TRACP-positive multinucleated cells was analyzed and expressed as average number of TRACP+ multinucleated cells per 5 micrographs for plastic or as number of TRACP+ multinucleated cells per cm^2 for bone. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are expressed as mean ± SEM in mono-cultures and co-cultures of cells from five different donors. For color figure see p.159.

The six hour pre-incubation with IL-1β led to a significant increase in fibroblast-free areas (Fig. 4B). Concomitantly, the number of $TRACP^+$ multinucleated cells was markedly increased. When dexamethasone was added, both parameters were inhibited (Fig. 4C).

Discussion

In this study we show that pre-incubation of periodontal ligament fibroblasts with IL-1β leads to a significant increase in the number of PBMCs adhered to the fibroblasts. This finding suggests that IL-1β can improve their proliferation, survival or adhesion to periodontal ligament fibroblasts. One likely candidate adhesion molecule responsible for this adhesion is ICAM-1, a cell adhesion molecule essential for the attachment of osteoclast precursors to osteoblast-like cells[5]. Here we showed that IL-1β stimulated the expression of ICAM-1. The observed increased expression of ICAM-1 is in line with data presented by others using osteoblasts [5] or periodontal ligament fibroblasts [17]. The enhanced expression of M-CSF due to IL-1β could then further induce osteoclast differentiation. After three days of co-culture no differences were observed anymore in ICAM-1 or M-CSF mRNA expression between the cultures. This indicates that the effects of IL-1β on mRNA expression of ICAM-1 and M-CSF are transient and disappear when the cytokine is no longer added. Together, these findings suggest that these cells can differentiate in a subpopulation that is more prone to support osteoclastogenesis under the influence of IL-1.

The unaffected RANKL mRNA expression and the high OPG expression as well as the unaltered RANKL/OPG ratio in the presence of IL-1β suggest that this subpopulation can regulate osteoclastogenesis, at least in part, in a RANKL-RANK-OPG independent manner.

IL-1β can thus shift the phenotype of osteoblast-like cells from one that participates in bone formation [23] to one that favors – albeit indirectly - bone destruction (this study).

IL-1β administration also significantly increased mRNA expression of the cytokine itself. This transcriptional auto-regulation of IL-1β may facilitate long term effects after a relatively short IL-1β stimulation and further promote activities among which osteoclast differentiation. The present study shows that the IL-1β auto-regulating signaling in periodontal ligament fibroblasts and PBMCs can be inhibited by dexamethasone.

At a later stage during osteoclastogenesis osteoblast-like cells retract and create a space for primed osteoclast precursors to migrate to the surface [9]. We demonstrate that an initial stimulation with IL-1β leads at a later time point (21 days) to an increased fibroblast-free area. Fibroblast migration can be induced either directly, via an effect of IL-1β on the fibroblasts themselves, or indirectly, via the PBMCs. These cells may exert a different response on stimulated fibroblasts and may stimulate migration of the latter cells. IL-1β-associated migration has also been proposed for other cell types [10,24,25]. In A549 epithelial cells, IL-1β induced the expression of matrix metalloproteinases which led to cell migration [10]. It is possible that in periodontal ligament fibroblasts IL-1β also stimulates cell migration through an increase in MMP expression since our group previously showed that (i) IL-1 stimulates the expression of MMPs by periodontal ligament fibroblasts [26,27], and (ii) the retraction of osteoblast-like cells is mediated by these proteinases [9]. In this regard MMP-3 (stromelysin-1) could be a good candidate. This enzyme can cleave homotypic interactions mediated by adhesion molecules such as E-cadherin [28] and it has been described that IL-1β increases the expression of MMP-3 in periodontal ligament fibroblasts at both mRNA and protein level [26].

We next examined the number of osteoclasts formed and this closely correlated with the surface area of fibroblast-free areas. Thus it appears that even such a short interaction (6h) between the cytokine and the fibroblasts results in changes at later stages of osteoclast differentiation.

In conclusion, our data indicate that IL-1β can favor osteoclastogenesis by changing the phenotypic characteristics of the supporting periodontal ligament fibroblasts already after a short and single exposure of these cells to the cytokine. This may have implications for the understanding of affected bone remodeling in periodontitis.

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